Use of MMTV-*Wnt-1* transgenic mice for studying the genetic basis of breast cancer

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Wnt-1 was first identified as a protooncogene activated by viral insertion in mouse mammary tumors. Transgenic expression of this gene using a mouse mammary tumor virus LTR enhancer causes extensive ductal hyperplasia early in life and mammary adenocarcinomas in approximately 50% of the female transgenic (TG) mice by 6 months of age. Metastasis to the lung and proximal lymph nodes is rare at the time tumors are detected but frequent after the removal of the primary neoplasm. The potent mitogenic effect mediated by *Wnt-1* expression does not require estrogen stimulation; tumors form after an increased latency in estrogen receptor α-null mice. Several genetic lesions, including inactivation of *p53* and over-expression of *Fgf-3*, collaborate with *Wnt-1* in leading to mammary tumors, but loss of *Sky* and inactivation of one allele of *Rb* do not affect the rate of tumor formation in *Wnt-1* TG mice. *Oncogene* (2000) 19, 1002–1009.

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Introduction

Infection of most strains of mice, such as C3H, with mouse mammary tumor virus (MMTV) leads to a high incidence of mammary tumors (reviewed by Nusse, 1991). The sites of insertions by MMTV proviruses have been extensively mined in order to identify genes that are deregulated to cause tumorigenesis. *Wnt-1* was the first protooncogene to be cloned following activation by viral insertion in mouse mammary tumors (Nusse and Varmus, 1982). (Its initial name, *int-1*, was subsequently changed to *Wnt-1* because of its homology to the Drosophila *Wingless (Wg)* gene (Nusse *et al.*, 1991)). Insertional activation of *Wnt-1* occurs in approximately 70% of C3H mice that are chronically infected with MMTV (Nusse and Varmus, 1982). Other candidate protooncogenes that are sometimes activated by MMTV proviral insertions include two additional members of the *Wnt* family, *Wnt-3* (Roelink *et al.*, 1990) and *Wnt-10b* (Lee *et al.*, 1995); three members of the fibroblast growth factor family, *Fgf-3/int-2* (Dickson *et al.*, 1984), *Fgf-4/hst* (Peters *et al.*, 1989), and *Fgf-8/AIGF* (MacArthur *et al*, 1995); *Notch-4/int-3* (Lee *et al.*, 1995); and *int-6* (Asano *et al.*, 1997), encoding a subunit of the translation initiation factor eIF3. Some of these genes, such as *Fgf-3* and *Wnt-10b*, have been validated as oncogenes by transgenic expression (Kwan *et al.*, 1992; Lane and Leder, 1997; Muller *et al.*, 1990).

The *Wnt-1* gene encodes a member of a large family of secreted proteins that are cysteine-rich, glycosylated, and poorly soluble (reviewed by Nusse and Varmus, 1992). Presently, at least 18 distinct Wnt family members have been identified in mammals. Having a propensity to associate with the extracellular matrix, Wnts act on both Wnt-producing and adjacent cells through cell surface receptors to control cell fate and patterning (reviewed by Nusse and Varmus, 1992). In mice, *Wnt-1* is expressed exclusively in the developing central nervous system (CNS) and adult testes (Jakobovits *et al.*, 1986; Shackleford and Varmus, 1987; Wilkinson *et al.*, 1987), and it is required for CNS patterning and development of the midbrain and cerebellum (McMahon and Bradley, 1990; Thomas and Capecchi, 1990). Its Drosophila ortholog, *Wg*, controls segment polarity and many other developmental processes (Wodarz and Nusse, 1998).

A brief overview of the Wnt signaling pathway

One of the major intracellular responses to Wnt-1 signaling is to stabilize and increase the level of cytosolic β-catenin (Figure 1), a multi-functional protein that associates with membrane-bound E-cadherin, as well as several DNA binding proteins, such as members of the TCF/LEF family (reviewed by Kinzler and Vogelstein, 1996). Heterodimers of β-catenin and transcriptions factors translocate to the nucleus and transactivate a number of genes, including *c-myc* (He *et al.*, 1998), *cyclin D1* (Shtutman *et al.*, 1999; Tetsu and McCormick, 1999), *WISPs* (Pennica *et al.*, 1998), and possibly *cyclooxygenase-2* (Howe *et al.*, 1999). Depending upon the cell type, Wnt signaling activates different genes, affecting various stages of development and several types of cancer.

The receptors for Wnts have been identified as a class of seven transmembrane proteins known as Frizzled (Fz) (Bhanot *et al.*, 1996). The ligand-receptor interaction is facilitated by extracellular proteoglycans and, inhibited by Fz-related proteins, dickkopf, and cerberus. After binding a Wnt ligand, Fz transmits a signal to cytoplasmic phosphoproteins in the disheveled (Dvl) family via unknown mechanisms. Dvl inhibits the constitutively active kinase activity of glycogen synthase kinase type 3 (GSK3), which normally phosphorylates β-catenin and targets it for degradation. Cytosolic levels of β-catenin are additionally regulated by adenomatous polyosis coli (APC), which targets β-catenin for proteosome-mediated degradation, and by another large protein, Axin, also an inhibitor of Wnt signaling. A complex

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containing APC, Axin, β-catenin, GSK3, and GSK-binding protein/Frat-1 has been observed in lysates prepared from certain cell types (reviewed by Barish and Williams, 1999).

Several members of the Wnt family transform cultured cells, when overexpressed. For example, overexpression of *Wnt-1, Wnt-2, Wnt-3*, and *Wnt-3a* (but not *Wnt-4, Wnt-5a, Wnt-5b*, and *Wnt-7b*) leads to morphological transformation of mammary epithelial cells such as C57MG (Brown *et al.*, 1986; Shimizu *et al.*, 1997). Continued overexpression is required for the transformation phenotype induced by *Wnt-1* (Li *et al.*, 1999; Mason *et al.*, 1992).

Wnt-1 is not normally expressed in the mammary gland, nor has it been directly implicated in human breast cancer. However, several other *Wnt* family members are expressed in breast tissue, and some are overexpressed in breast tumors (reviewed by Bergstein and Brown, 1999). In addition, genes encoding several components and targets of the Wnt signaling pathway, including β-catenin, APC, E-cadherin, cyclin D1, c-*myc*, and WISPs, have been found to be mutated or deregulated in several types of human tumors, such as breast cancer (Bieche *et al.*, 1999), colon cancer (He *et al.*, 1998), melanoma (Rimm *et al.*, 1999; Rubinfeld *et al.*, 1997), hepatocellular carcinoma (de La Coste *et al.*, 1998), and pilomatricoma (Chan *et al.*, 1999).

The Wnt-1 transgenic (TG) mouse model

Wnt-1 TG mice were initially made to test the oncogenicity of *Wnt-1* (Tsukamoto *et al.*, 1988). The transgene (Figure 2), is controlled by the *Wnt-1* promoter and an MMTV LTR inserted upstream of the gene in the opposite transcriptional orientation, in a fashion reminiscent of a typical viral insertion into the *Wnt-1* locus in MMTV-induced tumors. Ectopic *Wnt-1* expression exerts a potent mitogenic effect on mammary epithelium; ductal hyperplasia is noticeable in the mammary end-buds by 18 days of gestation (Cunha and Hom, 1996) and very apparent 2 weeks after birth in the TG females (Lin *et al.*, 1992). Because of the extensive ductal hyperplasia, female TG mice can not deliver milk to their young.

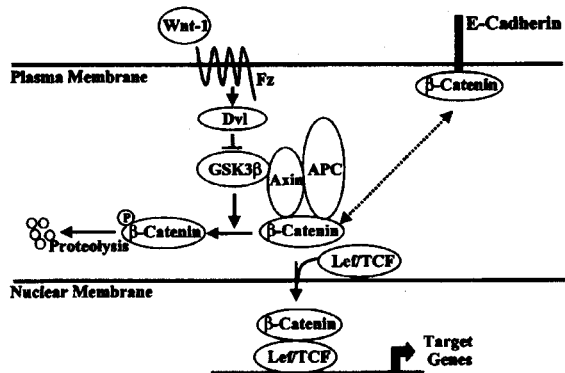About 50% of virgin female *Wnt-1* TG mice in the SJL strain develop adenocarcinomas by 6 months of age; the rest succumb to tumors by 1 year. Breeding females develop tumors slightly earlier than virgin mice (Shackleford *et al.*, 1993; Tsukamoto *et al.*, 1988). This acceleration may be caused by either hormonal influence on cell growth or the increased mass of the mammary epithelium attributed to pregnancies and lactation. Hyperplasia is also extensive in the primary mammary glands of adult male TG mice; about 15% of them develop palpable mammary tumors by 1 year of age (Kwan *et al.*, 1992; Tsukamoto *et al.*, 1988). Although metastasis does not seem to occur frequently at the time mammary tumors are detected (Tsukamoto *et al.*, 1988), the majority of female *Wnt-1* TG mice develop lymph node and/or lung metastasis after removal of the primary tumor (L Godley and WP Hively, unpublished observation).

Tumors found in Wnt-1 TG mice are usually moderately differentiated and comprised of ducts with multiple layers of epithelial cells, that show significantly higher than normal nucleus-to-cytoplasm ratio and occasionally pleomorphic nuclei and mitotic figures. The lumens usually contain pyknotic cells suggestive of apoptosis. Widespread necrosis and hemorrhage are sometimes noticeable in these tumors. In addition, extensive fibrosis is present in neoplasms induced by the *Wnt-1* transgene. Hyperplastic glands of *Wnt-1* TG mice also display a prominent fibrotic response, which may start as early as 7 days postnatally in TG females (G Cunha, personal communication).

Variations in genetic backgrounds usually do not influence the time course of tumor development mediated by the *Wnt-1* transgene (Bocchinfuso *et al.*, 1999; Donehower *et al.*, 1995; Shackleford *et al.*, 1993; Tsukamoto *et al.*, 1988). The original TG line was made in C57BL/6 X SJL F1 mice. Subsequently, interbreedings with other strains (FVB/N, BALB/c, 129/J, C58BL/6) have been found to be similar to SLJ in tumor latency (Table 1). But a much longer latency has been observed in some mixed backgrounds (C Alexander, personal communication).
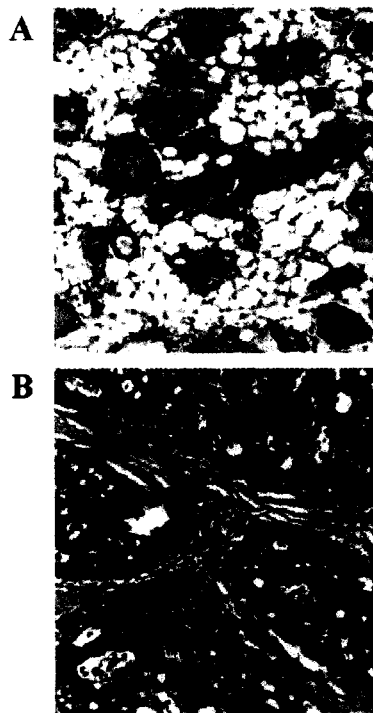
Figure 2 *Wnt-1* transgene construct. The 7 kb transgene contains the MMTV-LTR approximately 1 kb upstream of the mouse *Wnt-1* gene. The MMTV-LTR was placed in the opposite transcriptional orientation and is used as an enhancer. The *Wnt-1* coding sequences are shown as filled boxes. A fragment containing the SV-40 splice and polyadenylation sites (850 bp) was placed downstream of the last exon of *Wnt-1*

Table 1 Genetic lesions crossed to the MMTV-*Wnt-1* transgene

Genotype	Genetic background	References
MMTV-*Fgf-3* TG	FVB/N	Muller *et al.*, 1990; Kwan *et al.*, 1992
Sky −/−	129/Sv × C57BL/6	Lu *et al.*, 1999; WP Hively (unpublished)
p53 −/−	129/Sv	Donehower *et al.*, 1992, 1995
ERα −/−	C57BL/6	Lubahn *et al.*, 1993; Bocchinfuso *et al.*, 1999
MMTV-TGF	C57BL/6	A Chytil, Y-L Chen and HL Moses (unpublished)

Figure 1 Illustration of the Wnt signal transduction pathway (kindly provided by J-M Li). See text for explanation

Figure 3 Histology of mammary glands from an 18-week-old MMTV-*Wnt-1* TG virgin female. (a) Hyperplastic mammary gland (25 ×). (b) Mammary adenocarcinoma (25 ×)

Many genetic lesions and epigenetic changes, such as levels of mammogenic hormones, have been implicated in breast carcinogenesis. However, the complex molecular interplay leading to breast cancer is very poorly understood. All mammary epithelial cells expressing the transgene in the *Wnt-1* TG line are at risk for tumor development. Indeed, ductal hyperplasia occurs throughout the mammary tissue early in development, yet tumors appear stochastically after several months. Therefore, other cooperative events must have accompanied expression of the *Wnt-1* transgene in the few cells that expanded into tumors. A number of methods have been applied to uncover these synergistic events. Among them are hormonal manipulations, insertional activation of protooncogenes using retroviral infection, and breeding with TG and knockout mice carrying other genetic lesions implicated in breast cancer.

Hormonal and stromal influences on Wnt-1-*induced hyperplasia and tumors*

Estrogen is essential in mammary development and plays a very important role in carcinogenesis of the breast (reviewed by Pike *et al.*, 1993). It stimulates ductal morphogenesis and branching through nuclear receptors – ERα and possibly the recently identified ERβ (Kuiper *et al.*, 1996; Mosselman *et al.*, 1996). ERα is expressed in both mammary epithelium and the stroma (Daniel *et al.*, 1987). ERβ is detectable in mammary tissues at low levels, but its role in mammary proliferation remains elusive (Krege *et al.*, 1998).

Hyperplastic ductal growth in the *Wnt-1* TG animals persists despite estradiol deprivation due to ovariectomy (Bocchinfuso *et al.*, 1999; Lin *et al.*, 1992). Albeit delayed, tumors continue to form in ovariectomized mice (Bocchinfuso *et al.*, 1999), suggesting that *Wnt-1* does not require estrogen signaling for stimulating proliferation and inducing tumors. These results were confirmed and strengthened by experiments in which the MMTV-*Wnt-1* transgene was crossed into the ERα knockout (*ERKO*) mice. In homozygous *ERKO* mice, mammary glands are underdeveloped, with rudimentary ducts confined to the nipple area (Lubahn *et al.*, 1993). The presence of the *Wnt-1* transgene stimulated hyperplastic ductal growth in *ERKO* mice, and the females developed mammary tumors at twice the age of *Wnt-1* TG females with one or two intact ERα genes (Bocchinfuso *et al.*, 1999). It remains to be determined if the increased latency to tumor development observed in ovariectomized or *ERKO* mice is the result of reduced mass of mammary epithelium, the loss of cooperative functions of ER signaling in *Wnt-1*-induced oncogenesis, or both.

The majority of human breast tumors are ERα-positive and respond to anti-hormone therapy; however, most malignant tumors are ERα-negative (McGuire and Clark, 1985). Together with the fact that only a small percentage of mammary epithelial cells express ERα (Petersen *et al.*, 1987; Ricketts *et al.*, 1991), it has been suggested that breast cancer may initiate from ER-positive cells but become ER-negative and estrogen-independent in its growth at later stages (Moolgavkar *et al.*, 1980). The observation that mammary tumors arise in both *ERKO* and ovariectomized mice supports an alternative model that a fraction of breast cancers may directly evolve from ERα-negative cells (Nandi *et al.*, 1995), an idea that needs to be tested with other oncogenic transgenes.

The potent mitogenic effect of Wnt-1 on mammary epithelial cells may not depend upon other mammogenic hormones either. For example, a similar degree of abnormal side branching was observed in mammary epithelial transplants derived from *Wnt-1* TG mice that were either wild-type or nullizygous for progesterone receptor α (*PRα*, C Brisken and R Weinberg, personal communication). Likewise, in ovariectomized and/or adrenalectomized mice, Wnt-1 continued to stimulate hyperplastic growth in transplanted and reconstituted glands (Edwards *et al.*, 1992; Lin *et al.*, 1992).

The reciprocal interactions between parenchyma and stroma are important in mammary development, remodeling, and carcinogenesis (reviewed by Cunha and Hom, 1996). For example, signaling through the epidermal growth factor receptor (EGFR) in the mesenchyme is required for ductal growth and branching morphogenesis, since epithelium transplanted from wild-type mice fails to proliferate in the fat-pad from *EGFR*-null mice (Wiesen *et al.*, 1999). Interestingly, this requirement also seems to be diminished in *Wnt-1* TG mice. Transplantation of epithelium from *Wnt-1* TG animals into the fat-pad of *EGFR* nullizygous mice only modestly impaired hyperplastic growth (G Cunha, personal communication). Epithelial-stromal interactions in tumor formation have also been studied by experiments in which mammary epithelial cells from *Wnt-1* TG animals were transplanted into rat mammary fat-pad. Transplanta-

tion led to fibrotic proliferation in rat mesenchyme (G Cunha, personal communication), suggesting that the alteration of stromal differentiation is mediated by the *Wnt-1*-expressing epithelial cells. Wnt-mediated epithelial-mesenchymal interactions have also been reported in other tissues. For example, Wnt-induced mesenchymal reactions may regulate axonal growth and guidance in developing limbs. Several members of the *Wnt* family expressed in limb ectoderm induce production of neurotrophin-3 in the underlying mesenchyme (Patapoutian *et al.*, 1999).

Collaboration between Wnt-1 *and other genes in oncogenesis*

Mammary tumors induced by MMTV occasionally show transcriptional activation of both *Wnt-1* and *fibroblast growth factor3* (*Fgf3*, Peters *et al.*, 1986), suggesting that these two genes collaborate in oncogenesis. Fgf3 belongs to a family of heparin-binding proteins that are both mitogenic and angiogenic. Signaling by FGFs is mediated by transmembrane receptors (FGFRs) that phosphorylate and activate several substrates, leading to the activation of mitogen-activated protein kinases (reviewed by Faham *et al.*, 1998). Although Wnts and FGFs act through very different pathways, they are both required for development of primary body axis, neural axis, limbs, and other structures, suggesting that these two families of growth factors may collaborate in development in ways that resemble synergistic roles in tumor formation.

Transgenic female mice expressing MMTV-*Fgf3* show extensive mammary hyperplasia but rarely develop tumors (Muller *et al.*, 1990). When this transgene was bred into *Wnt-1* TG mice, tumors developed faster in bi-transgenic females than in females bearing either transgene alone, providing direct evidence of cooperation between these two growth factors (Kwan *et al.*, 1992). The acceleration is even more dramatic in the bi-transgenic males. Additional evidence of synergistic interactions between *Wnt-1* and members of the *Fgf* family comes from infection of *Wnt-1* TG animals with MMTV (Shackleford *et al.*, 1993). Infection accelerates tumor formation, and up to ten tumors per mouse were observed in infected animals. Approximately 40% of the mammary tumors showed insertional activation of *Fgf3*, a small percentage of them had insertional activation of both *Fgf3* and *Fgf-4* or *Fgf4* alone. Another member of the *Fgf* family, *Fgf-8*, was also found to be insertionally-activated and/or overexpressed in some of these tumors (Kapoun and Shackleford, 1997; MacArthur *et al.*, 1995). Collaboration between members of the Wnt and FGF families has also been observed in experiments in which infection of MMTV-*Fgf3* TG mice with MMTV led to frequent viral insertions in *Wnt-1* or *Wnt-10b* loci (Lee *et al.*, 1995).

Tumor growth factor β (TGFβ) stimulates cell growth under some conditions, but, more commonly, inhibits cell proliferation, especially in the mammary gland (reviewed by Massague, 1998). For example, transgenic expression of *TGFβ* inhibits tumor formation in mice expressing an MMTV-*TGFα* transgene (Pierce *et al.*, 1995). But in a recent cross between our MMTV-*Wnt-1* TG mice and MMTV-*TGFβ* TG animals, no effects were observed on the rate of tumor appearance, histology, or the size of the tumor induced by the *Wnt-1* transgene (A Chytil, Y-L Chen and HL Moses, personal communication). Assuming adequate levels of expression, it appears TGFβ cannot inhibit proliferation of mammary epithelia stimulated by the *Wnt-1* transgene.

Collaboration between Wnt-1 *and loss of a tumor suppressor gene*

Several tumor suppressor genes are mutated or downregulated in human breast cancer. Inherited mutations of some of them predispose to breast neoplasm. For example, mutations of *BRCA-1* and *BRCA-2* are found in approximately 50% and 30%, respectively, of families predisposed to breast cancer (Ford *et al.*, 1998). Somatic *p53* mutations are found in about 35% of sporadic and 85% of familial breast cancers (Crook *et al.*, 1998), and germline alterations of *p53* are associated with a predisposition to several cancers, including breast cancer (the Li-Fraumeni syndrome). *RB*, a cell cycle regulator, is also mutated in a small percentage of sporadic human breast cancers (Berns *et al.*, 1995). In addition, p21/WAF1/CIP1, a cyclin-dependent kinase inhibitor that regulates G1-S cell cycle progression, is downregulated in some breast tumors, especially those with poor prognosis (Jiang *et al.*, 1997; Wakasugi *et al.*, 1997).

The impact of the loss of a tumor suppressor gene on tumorigenesis has been documented in animal models using targeted gene disruption, loss of heterozygosity (LOH) assays, and transgenic overexpression of a dominant-negative version of a tumor suppressor gene. Mice deficient for the *p53* tumor suppressor gene (*p53*+/− and *p53*−/−) develop tumors of non-epithelial origin (Donehower *et al.*, 1992). To analyse the effect of *p53* inactivation on mammary oncogenesis, *p53* knockout mice were bred with *Wnt-1* TG mice (Donehower *et al.*, 1995). *p53* nullizygotes (both females and males) expressing the *Wnt-1* transgene develop mammary tumors much earlier than mice containing at least one wild-type allele, suggesting that inactivation of *p53* plays an important role and collaborates with *Wnt-1* in mammary oncogenesis. In addition, *p53*-null tumors are more anaplastic and less fibrotic than tumors that carry at least one copy of the *p53* gene (Donehower *et al.*, 1995).

Although the absence of one copy of *p53* did not significantly alter the time at which MMTV-*Wnt-1* transgene induced tumors appeared, approximately 50% of the tumors in *p53*-heterozygous, *Wnt-1* TG mice displayed loss of the wild-type locus. This frequent occurrence of LOH contrasts with the very rare loss of the wild-type *p53* allele in mammary tumors from *p53* heterozygotes carrying an MMTV-c-*myc* transgene (Elson *et al.*, 1995; McCormack *et al.*, 1998). It is notable that inactivation of *p53* collaborates with MMTV-c-*myc*, MMTV-H-*ras*, and MMTV-*neu* transgenes to produce lymphomas and salivary tumors, but rarely mammary tumors (C-X Deng, personal communication, Elson *et al.*, 1995; Hundley *et al.*, 1997).

Mutations in *BRCA-1* and *BRCA-2* are often associated with loss of *p53* in breast carcinogenesis in humans (Crook *et al.*, 1998). Induction of mammary

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tumors in the mouse by mammary-specific *Brca1* inactivation is dramatically accelerated by inactivation of *p53* (Xu *et al.*, 1999). However, loss of one allele of *Brca-1* (T Wynshaw-Boris, personal communication) or *Brca-2* (XS Cui and LA Donehower, personal communication) does not seem to influence the kinetics of tumor formation induced by the *Wnt-1* transgene. It remains to be determined whether tumors that are heterozygous for *Brca1* or *Brca2* show LOH or alteration in karyotype. The availability of mice that carry loxP-flanked (floxed) alleles of *Brca1* and *Brca2* will permit better tests for synergy between the loss of *Brca1* or *Brca2* and inheritance of a *Wnt-1* transgene in tumor formation.

Germline mutations in one copy of the *Rb* gene predispose humans to retinoblastomas and osteosarcomas. Mice nullizygous for *Rb* die during embryogenesis; heterozygotes develop tumors primarily in the pituitary and thyroid glands but rarely in mammary glands (Jacks *et al.*, 1992). To determine if the loss of *Rb* affects the development of tumors in *Wnt-1* TG animals, we have crossed *Wnt-1* TG animals with mice heterozygous for *Rb*. Absence of one allele of *Rb* did not affect the age at which the tumor was detected, and none of 25 tumors examined by restriction mapping showed loss of the wild-type locus (WP Hively, unpublished). The lack of acceleration may be due to the complementary expression of one or both of the other two members of the *Rb* gene family (*p107* and *p130*) in the mouse mammary gland. In fact, the presence of normal *p107* alleles has been shown to inhibit *Rb* deficiency-mediated tumor formation in the mouse retina (Robanus-Maandag *et al.*, 1998). Elimination of all three members of the *Rb* gene family in *Wnt-1* TG mice would further clarify the role of their inactivation in mammary oncogenesis. One way to eliminate their functions is to generate transgenic mice expressing the gene encoding the amino terminal domain (T_{121}) of the Simian virus 40 T antigen, which inactivates all three members of the Rb family (Saenz Robles *et al.*, 1994; Symonds *et al.*, 1994).

Inactivation of one or both alleles of *p21* did not accelerate tumor formation in *Wnt-1* TG mice (Jones *et al.*, 1999). But, interestingly, tumors from *p21+/−* mice grew significantly faster, with a higher mitotic index and increased cyclin D1-associated phosphorylation of Rb, than those from either *p21+/+* or *p21−/−* mice (Jones *et al.*, 1999).

Additional tumor suppressor genes that collaborate with the *Wnt-1* transgene to induce tumor formation may be identified by scanning the whole genome for LOH. Application of this approach has led to the identification of two regions on mouse chromosomes 9 and 16 that are frequently deleted in insulinomas and carcinoid tumors in TG mice expressing the Simian virus 40 *large T antigen* (Dietrich *et al.*, 1994). Furthermore, using this technology in F1 hybrid mice between FVB/N and *Mus musculus castaneus*, Radany and colleagues (1997) have found that a marker on chromosome 4 from *Mus musculus castaneus* was frequently lost in MMTV-*H-Ras* transgene-induced mammary tumors. In contrast, no single chromosome was preferentially lost in tumors occurring in F1 progeny of a similar cross between Wnt-1 TG SJL mice and *Mus musculus castaneus* (unpublished data of K Hong *et al.*, cited in Radany *et al.*, 1997).

Molecular characterization of tumors from Wnt-1 TG animals

Chromosomal rearrangements including aneuploidy, chromosomal translocations and duplications, and amplification of selected genes are common in tumor cells (reviewed by Wright, 1999). Loss of *p53* function frequently leads to deregulated cell cycle control and chromosomal instability, which favors tumor growth (reviewed by Prives and Hall, 1999). Mammary tumors from *Wnt-1* TG mice with one or two functional copies of *p53* display occasional chromosomal abnormalities as shown by comparative genome hybridization (CGH, Kallioniemi *et al.*, 1992), which detects regions of expansion and deletion in all chromosomes. As expected, *Wnt-1* induced tumors without any *p53* function usually have more than one chromosomal abnormality. Tumors that arose in *p53* heterozygotes and experienced LOH at the *p53* locus displayed even more extensive alterations (at least three regions of DNA gain or loss) (Donehower *et al.*, 1995).

In general, it is difficult to anticipate what specific genes in an amplified chromosomal region may have synergized with an oncogenic transgene to induce neoplasm. But the distal region of chromosome 7, which was amplified in a *Wnt-1*-induced *p53−/−* tumor, is the site of *Fgf3* (Donehower *et al.*, 1995). Molecular hybridization using an *Fgf3*-specific probe confirmed that *Ffg3* was amplified and abundantly expressed in this tumor (Donehower *et al.*, 1995). This is different from human breast cancer, in which the syntenic region of chromosome 8q is frequently amplified (Brison, 1993; Lammie *et al.*, 1991; Theillet *et al.*, 1989), but *Fgf3* mRNA is not detected (Penault-Llorca *et al.*, 1995); however, a linked gene, *PRAD-1/CycinD1*, is usually overexpressed in such tumors (Motokura *et al.*, 1991).

Spectral karyotyping (SKY) labels each chromosome with a different color, allowing detection of chromosomal translocations and duplications (Liyanage *et al.*, 1996). We have analysed some tumors from *Wnt-1* TG mice that were *p53+/−* or *p53−/−*. Translocations, trisomy, and aneuploidy have been detected in cells cultured from some of these tumors (Z Weaver and WP Hively, unpublished). Karyotype instability in mammary tumors has been reported in mammary-specific *Brca1* knockout mice (Xu *et al.*, 1999) and other transgenic models including MMTV-c-*myc* (McCormack *et al.*, 1998; Weaver *et al.*, 1999).

As a physiologic response to genotoxins, *p53* is rapidly induced to cause cell cycle arrest and/or apoptosis. Inactivation of *p53* is often accompanied by accelerated cell growth and attenuated apoptosis (reviewed by Ko and Prives, 1996). *p53* deficiency (*p53+/−*, *p53−/−*) enhances cell proliferation in the *Wnt-1* transgene-derived tumors, but the modestly ongoing apoptosis that accompanies *Wnt-1* overexpression does not seem to be attenuated (Jones *et al.*, 1997). Similarly, absence of one allele of *p53* does not affect the apoptotic index in mammary tumors induced by an MMTV-c-*myc* transgene (McCormack *et al.*, 1998).

Normal telomeres are essential to cell survival. Telomerase is usually activated in human cancer cells, presumably to overcome shortened telomeres due to excessive cell replication (reviewed by de Lange and DePinho, 1999). Normal telomeres (20–50 kb) are

present in both hyperplastic glands and carcinomas from *Wnt-1* TG mice, regardless of the *p53* status (Broccoli *et al.*, 1996). Interestingly, despite the presence of long telomeres, telomerase activity and the RNA component of the enzyme were consistently upregulated in these tumors compared with normal and hyperplastic glands (Broccoli *et al.*, 1996), suggesting that activation of the telomerase machinery in at least some mammary tumors does not depend upon telomeric shortenings. Breeding *Wnt-1* TG animals with mice that carry a mutated gene for telomerase or a component of the telomeric complex (Blasco *et al.*, 1997; Rudolph *et al.*, 1999) will help address whether the telomere activation is required for mammary oncogenesis induced by the *Wnt-1* transgene.

Another approach to uncovering the molecular basis of tumorigenesis is to identify differentially-expressed genes during various stages of tumor formation. Several methods including subtractive hybridization, differential display, serial analysis of gene expression (SAGE), and cDNA expression array technology have been used. A number of genes have been found to be deregulated in *Wnt-1*-induced tumors and *Wnt-1*-transformed cells by these and other methods. For example, using PCR to screen for differentially expressed tyrosine kinases, we found that *Sky*, which encodes a member of the Axl/Ufo family of receptor tyrosine kinases, is barely detectable in the mammary glands from virgin animals and in preneoplastic mammary glands, but is abundantly expressed in the mammary tumors of *Wnt-1* TG mice (Taylor *et al.*, 1995). Recently, using a modified subtractive hybridization approach, Pennica *et al.* (1998) have found that two novel genes, *WISP1* and 2, are overexpressed in *Wnt-1*-transformed mammary cells and that they are transcriptionally regulated by *Wnt-1* expression and aberrantly expressed in colon cancer.

Screening for upregulated genes in tumors might also help identify collaborating factors in tumor formation. But alteration of the transcriptional apparatus during neoplastic conversion may deregulate many non-collaborating genes. An example of such a non-synergistic element is *Sky*. Tumor development in *Wnt-1* transgenic mice was not affected by breeding *Sky* knockout mice (Lu *et al.*, 1999) with *Wnt-1* TG animals (WP Hively, unpublished), suggesting that overexpression of *Sky* is not necessary for *Wnt-1* mediated oncogenesis.

Involvement of other components of the Wnt signaling pathway in mammary carcinogenesis

Many other components of the Wnt signaling pathway have been implicated in mammary tumorigenesis. Mutations in *APC*, a negative regulator of the Wnt signaling pathway, have been reported to confer an increased risk for development of breast cancer in Ashkenzi Jews (Redston *et al.*, 1998; Woodage *et al.*, 1998). *Min* mice, which carry a nonsense mutation at one *APC* locus, also have increased risk for mammary carcinomas after carcinogen treatment (Moser *et al.*, 1993, 1995). With the use of additional TG mice, it will be interesting to determine if deregulated expression of other components of the Wnt-1 signaling pathway, such as over-expression of β-

catenin and inactivation of *E-cadherin*, also induce mammary tumors.

All three members of the *Dvl* family (*Dvl1*, *Dvl2* and *Dvl3*) are expressed in mammary glands, with *Dvl1* being most abundant (Tsang *et al.*, 1996). Dvl proteins transmit signals from the Fz receptor for Wnt-1 to β-catenin via unknown mechanisms (see Introduction). Mice nullizygous for *Dvl1* are normal except for abnormalities in social behavior and sensorimotor gating (Lijam *et al.*, 1997). The mammary epithelia lacking the dominant member of this family might be expected to respond poorly to *Wnt-1* induced cell proliferation and tumor formation. But *Dvl1* nullizygosity did not affect the rate of tumor formation in *Wnt-1* TG mice (N Lijam, WP Hively, HE Varmus and T Wynshaw-Boris, unpublished). Since *Dvl2* and *Dvl3* are also expressed in the mammary gland, they might have substituted for *Dvl1* in mediating the Wnt-1 signal.

Syndecan-1 is a member of the transmembrane proteoglycan family that regulates cell morphology and growth (Leppa *et al.*, 1992). Proteoglycans facilitate the binding of Wnt ligands to Fz receptors (Lin and Perrimon, 1999; Wodarz and Nusse, 1998). Consistent with this finding, *Wnt-1* TG mice that carry two null alleles of *syndecan-1* very rarely develop tumors (C Alexander, personal communication), suggesting that syndecan-1 may be an important factor in mediating Wnt-1 signaling in the mammary gland.

Prospects

Although initially developed to document the oncogenic potential of *Wnt-1*, our line of MMTV-*Wnt-1* TG mice has been useful in studying many aspects of mammary tumorigenesis: the cooperation between cancer genes, the influence of estrogen receptors and growth hormones, and the concomitant changes in genomic instability and gene expression.

Different stages of tumor progression can be discerned in *Wnt-1* TG mice, and some of the collaborative lesions accompanying *Wnt-1* overexpression in tumor formation have been defined. Therefore, this line may be a convenient source of hyperplastic glands and invasive and metastatic tumors for various approaches designed to identify molecular signatures of tumor progression. Comparing the expression profile of the *Wnt-1*-derived tumors with those of tumors derived from *Wnt-1* TG mice crossed with other genetically modified lines may offer additional insights into the complex nature of mammary oncogenesis. Additional benefits in the characterization of these tumors include identification of transcriptional targets of the Wnt-1 signaling pathway.

Recently, a novel method has been used to transduce oncogenes into somatic cells of a specific tissue (reviewed by Fisher *et al.*, 1999) using sub-group A avian leukosis virus (ALV-A) as a vector. Transgenic expression of *tv-a*, encoding the receptor for ALV-A, from a cell type-specific promoter, permits tissue-specific infection with ALV-A, which does not produce infectious virus in mammalian hosts. Consequently, combinatorial effects of genetic lesions can be examined in a single TG line by infecting with mixtures of ALV-A viruses expressing different oncogenes. In addition, ALV-A expressing the gene encoding Cre

recombinase can be used to inactivate tumor suppressor genes flanked with loxP recombination sites. Since ALV infection requires mitotic cells which are widely available only during late pregnancy, breeding the *Wnt-1* transgene into mice expressing *tv-a* from a mammary-specific promoter may provide both replicating epithelial cells (eliminating the requirement for pregnancies) and a cancer predisposing factor allowing more rapid formation of tumors. The TVA technology may help test candidate collaborative events in context of the *Wnt-1* transgene.

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